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Association of β1 integrin with protein kinase activity in large detergent resistant complexes

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Abstract Integrins play a critical role in cell adhesion and mediate cell signaling. This report identifies the association of serine protein kinase activity with the β1 integrin by immunoprecipitation and phosphoamino acid analysis techniques. Reprecipitation techniques suggested that the serine kinase activity was not a member of the protein kinase C family. By gel filtration, most of the protein kinase activity associated with β1 integrin as well as most of the cell-surface β1 integrin was present in large detergent resistant complexes. These results suggest that serine protein kinase activity associated with the β1 integrin may play a role in signaling via the β1 integrin.

Key words: β1 Integrin; Protein kinase activity; Detergent resistant complex; Ovarian carcinoma

1. Introduction

Integrins are the major family of receptors that mediate the adherence of epithelial cells to basement membrane components [1]. Integrins are comprised of an α and a β subunit that are non-covalently associated. Integrins have been shown to be involved in signal transduction, whereby 'inside-out' and 'outside-in' signaling occurs via integrins interacting with a variety of extracellular and intracellular molecules (reviewed in [2–4]), many of which still remain to be elucidated. Laminin and type IV collagen, the two major basement membrane proteins, have been shown to promote cell adhesion, spreading, migration, and invasion, and also alter the growth, development, and differentiation, of a variety of cell types [5,6]. In addition, the expression of laminin and integrins is altered in a number of malignancies (reviewed in [7]). The exact mechanism of integrin-mediated signal transduction remains unclear.

Phosphorylation has been shown to be an important mechanism of regulating protein function in a variety of systems, including the functioning of integrin subunits [2–4]. Recently, integrin-mediated cell adhesion has been shown to result in tyrosine phosphorylation of focal adhesion kinase, which then may initiate a series of phosphorylation events including the binding of Src [2]. Integrin-mediated cell adhesion may also result in the phosphorylation of the intracellular proteins tensin, paxillin, and p130 [8–10]. At focal adhesion sites, where cells are in contact with the extracellular matrix, integrins, cytoskeletal proteins, and kinases have been localized. In particular, focal adhesion kinase, protein kinase C, and cSrc have been implicated as important kinases that work with integrins to mediate a variety of cell functions. Some of these functions include cell proliferation, cell differentiation, and apoptosis [2]. In addition, protein kinase activity has been found to be associated with a variety of membrane proteins involved in signal transmission, including CD50, CD63, and CD66, [11–14]. Recent studies have demonstrated the existence of large detergent resistant complexes in cell extracts that contain important signaling molecules, including protein kinases and membrane proteins capable of transmitting signals [15–22].

To address whether the β_1 integrin subunit is associated with protein kinase activity, four ovarian cancer cell lines and one teratocarcinoma cell line were analyzed for the expression of β_1 integrin, and for integrin associated protein kinase activity. By flow cytometry these cell lines were found to express high levels of the α_3 and β_1 integrin subunits, while the overall expression of other integrin subunits such as α_1, α_2, α_6, and β_4 was, in general, moderate or low. When the β_1 integrin was immunoprecipitated from the solubilized cells, protein serine kinase activity associated with the integrin was detected. Although protein kinase C (PKC) activity was present in these cells, it was not present or could not be detected in the β_1 integrin immunoprecipitates. The data suggest that phosphorylation of cellular proteins by an associated serine kinase, other than PKC, may play a role in signal transduction by β_1 integrins. In addition, studies using gel filtration chromatography demonstrated that most of the cell surface β_1 integrin, as well as the associated kinase activity, was present in large detergent resistant complexes.

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Abbreviations: DFP, diisopropylfluorophosphate; NMS, normal mouse serum; NaCl-HEPES, 145 mM NaCl, 20 mM HEPES, pH 7.3; HBSS, Hanks' balanced salt solution; Cell solubilization buffer, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% NP-40, 0.02% NaN_3, and 1 mM PMSF; Brij solubilization buffer, 20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 1 mM PMSF, 2 mM $MgCl_2$, 0.02% NaN_3, and 1.0% Brij 58; Brij wash buffer, 20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 1 mg/ml BSA, 0.5% Brij 58, 2 mM $MgCl_2$, 0.125 mg/ml gelatin, 1 mM PMSF, and 0.02% NaN_3; Labeling buffer, NaCl-HEPES, 0.1% Brij 58, 6 mM $MnCl_2$, 40 mM $MgCl_2$, 200 μM Na_3VO_4, 200 μM Na_2MoO_4, and 10 μCi of [γ-^{32}P]ATP; SA buffer, 1 mg/ml BSA, 0.05% NP-40, 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.05% NaN_3

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2. Materials and methods

2.1. Cell lines

Four ovarian carcinoma cell lines derived from the ascites fluid of patients with ovarian carcinoma were used in this study, as well as one teratocarcinoma cell line, PA-1, that had metastasized to the ovary. All of the cell lines were purchased from the American Type Culture Collection except for the OVCA 433 cell line that was generously provided by Dr. Robert C. Bast, Jr. (University of Texas, Houston, TX). The SKOV3 cell line was grown in McCoy's 5A media containing 0.03% L-glutamine, penicillin, streptomycin, and 15% heat inactivated fetal bovine serum. The PA-1 cell line was grown in MEM containing non-essential amino acids, Earle's Basic Salt Solution and 10% heat inactivated fetal bovine serum. The OVCA 433 cell line was grown in MEM containing 0.03% L-glutamine, 2 mM sodium pyruvate, non-essential amino acids, penicillin, streptomycin, and 10% heat inactivated fetal bovine serum. The SW626 cell line was grown in Leibovitz 2-15 media containing 10% fetal bovine serum. The CAOV-3 cell line was grown in DMEM containing 4.5 g/l glucose and 10% fetal bovine serum. All of the cell lines were grown in a 37°C humidified incubator containing 5% carbon dioxide, except the SW626 cell line which was grown in an incubator without carbon dioxide. Once the cell lines were thawed, they were grown for less than eight passages in order to prevent phenotypic drift. The antibiotic concentrations were 100 U/ml for penicillin and 100 μg/ml for streptomycin (Sigma). Cells were suspended at the indicated concentrations in 145 mM NaCl, 20 mM HEPES (Gibco, Grand Island, NY), pH 7.3 (NaCl-HEPES). Viability as assessed by trypan blue dye exclusion was greater than 98%.

2.2. Antibodies and reagents

Culture supernatant of the CD29 mAb P5D2 (anti-β1 integrin) was provided by Dr. Leo Furcht (University of Minnesota, Minneapolis, MN) [23]. An mAb against CA-125, termed OC 125, was provided as ascites by Dr. R.C. Bast, Jr. and was also purchased from Signet Laboratories, Inc. (Dedham, MA) as purified IgG1. An mAb against the 185-kDa c-neu protein, termed TA-1, was purchased from Oncogene Science (Uniondale, NY) as purified IgG1. The anti-α1 mAb TS2/7 [24] was purchased from T Cell Diagnostics (Cambridge, MA). The anti-β1 mAb P4C10, the anti-β4 mAb 3E1, the anti-α2 mAb P1E6, the anti-α3 mAb P1B5, and the anti-α4 mAb P4G9 were purchased from Life Technologies, Gibco/BRL (Grand Island, NY). Four anti-α3 mAbs, ASC-1, ASC-5, ASC-6, and ASC-10, as well as four anti-β4 mAbs, ASC-3, ASC-4, ASC-8, and ASC-9 were produced as previously described [25]. Culture supernatant of the anti-α6 mAb GOH3 was generously provided by Dr. Arnoud Sonnenberg from the Netherlands Cancer Institute, Amsterdam [26,27] or was purchased from AMAC (Westbrook, ME). All sera and ascites were heat inactivated at 56°C for 30 min and clarified by centrifugation at $13\,000\times g$ at 4°C for 30 min before use. Antibodies to the protein kinase C members alpha, beta, gamma, delta, and epsilon, were obtained from Promega (Madison, WI). Purified mouse IgG and normal mouse serum (NMS) were purchased from Sigma Chemical Co. (St. Louis, MO). Other negative controls included spent media from the Sp2/0-Ag14 myeloma cell line that was purchased from the American Type Culture Collection.

2.3. Flow cytometry analysis

Cells were analyzed for the expression of the various integrin subunits on their surface by flow cytometry as previously described [25]. Approximately 10^4 fluorescent events were counted in order to determine the mean fluorescent intensity (MFI) for each sample.

2.4. Preparation of biotinylated cell extract

Cells (5×10^6 cell/ml) in PBS were incubated with 100 μg/ml of sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce, Rockford, IL) for 30 min at room temperature on a rocker platform in the dark and washed twice with PBS [13]. Biotinylated cells were then solubilized and used for immunoprecipitation as described below.

2.5. Immunoprecipitation and PAGE

Immunoprecipitation was performed as previously described with minor modifications [11,13]. For ^{32}P-labeled proteins, gel slabs were stained, dried, and subjected to autoradiography by using Dupont Cronex film. For biotinylated proteins, proteins were resolved by SDS-PAGE and then transferred by electroblotting to Immobilon transfer membranes (Millipore Corp., Bedford, MA) as previously described [28] with a Bio-Rad Trans-Blot transfer cell at 30 V for 12 h at 4°C and then at 80 V for 2 h at 4°C. Protein transfer was monitored by amido black staining [29]. After transblotting, the nitrocellulose was blocked by incubation in PBS containing 3% BSA for 4 h at 4°C and washed four times with PBS. Biotinylated proteins on the blot were visualized by using the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL) following the manufacturer's protocol. After incubation with the ECL reagents, the blot was exposed to Hyperfilm-ECL (Amersham).

2.6. Protein kinase assay

Cell proteins in cell solubilization buffer were immunoprecipitated as described previously [11,13]. Cell proteins in Brij solubilization buffer were immunoprecipitated as described above except the immunoprecipitation mixture contained 0.5% Brij 58, 1 mg/ml BSA, 20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 2 mM $MgCl_2$, 0.125 mg/ml gelatin, and 1 mM PMSF, and the immunoprecipitates were washed three times with Brij wash buffer (20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 1 mg/ml BSA, 0.5% Brij 58, 2 mM $MgCl_2$, 0.125 mg/ml gelatin, 1 mM PMSF, and 0.02% NaN_3) and then once with NaCl-HEPES. Immunoprecipitates were suspended in 30 μl of NaCl-HEPES. Thirty μl of labeling buffer (NaCl-HEPES, 0.1% Brij 58, 6 mM $MnCl_2$, 40 mM $MgCl_2$, 200 μM Na_3VO_4, 200 μM Na_2MoO_4, and 10 μCi of [γ-^{32}P]ATP) was then added, and the mixtures were incubated for 10 min at 23°C. The reaction was stopped by adding 4× Laemmli sample buffer containing 400 μM ATP, incubated at 100°C for 2 min, and analyzed by SDS-PAGE and autoradiography as described above.

2.7. Identification of protein kinase C isozymes

To identify specific protein kinase C (PKC) isozymes associated with the β1 integrin, cells were solubilized in Brij solubilization buffer, immunoprecipitated with the indicated mAb, and the immunoprecipitates were radiolabeled with [γ-^{32}P]ATP as described above. The precipitate was solubilized with 200 μl of NaCl-HEPES containing 1% SDS, 200 μM Na_3VO_4, and 200 μM Na_2MoO_4 for 10 min at 23°C, and immunoprecipitated as described previously [11,13].

A second technique was also used to identify PKC isozymes associated with the β1 integrin. Cell extracts were immunoprecipitated as described above and the immunoprecipitates were analyzed by immunoblotting with antibodies specific to various PKC isozymes. Immunoblotting was performed as previously described [29]. After transfer of proteins onto PVDF, the membranes were blocked by incubation in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, containing 0.1% Tween-20 (Sigma) and 0.04% NaN_3 (T-TBS), with 5% NGS (Gibco), overnight at 23°C with constant rocking. After two washes with T-TBS, PVDF strips were incubated for 2 h at 23°C in T-TBS/5% NGS, containing 2 μl of mouse ascites per 2 ml of T-TBS/5% NGS, on a rocker platform. The first antibody was removed and the strips were washed three times with T-TBS. The strips were then incubated for 1 h at 23°C with biotinylated goat anti-mouse IgG (H and L chain specific: Organon Teknika Corp., West Chester, PA) diluted 1:1000 in T-TBS/5% NGS (2 ml/strip). The second antibody was removed, and the PVDF was washed three times in T-TBS and then incubated for 30 min with avidin-conjugated alkaline phosphatase (Organon Teknika Corp) diluted 1:5000 in T-TBS/5% NGS (5 ml/well). After two T-TBS washes, proteins were visualized by incubation in 0.3 mg/ml nitroblue tetrazolium (Sigma), 0.2 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Sigma), 100 mM NaCl, 50 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.5, for 30 min at 23°C in the dark. The reaction was stopped by washing in distilled water, at 23°C.

2.8. Gel chromatography

Gel filtration chromatography was performed as previously described [30]. Briefly, 3-ml columns (0.8×5 cm) of Sepharose 4B (Pharmacia) were equilibrated with Brij solubilization buffer at 23°C, and 0.3 ml of cell extract was applied to the top of the column. The column was eluted with Brij solubilization buffer, and 0.3 ml fractions were collected. The column was calibrated with Blue Dextran (void volume) (Sigma), which eluted in fractions 2 and 3, and IgG, which eluted in fraction 7.

2.9. Phosphoamino acid analysis

Phosphoamino acid analyses were performed as previously de-

scribed [11,12,31]. The radiolabeled phosphoamino acids were detected by autoradiography using X-Omat AR film.

3. Results

3.1. Expression of integrin subunits on the surface of cells

Flow cytometry analysis was performed in order to determine the repertoire of integrin subunits that were present on the surface of four of the cell lines that were used in this study. The SKOV3, CAOV-3, OVCA433, and PA-1 cell lines expressed very high levels of α3 and β1 integrin subunits on their surface as determined by flow cytometry (data not shown). In addition, all four cell lines cells expressed moderate to low levels of the α1, α2, α6, and β4 integrin subunits (data not shown).

3.2. Identification of protein kinase activity associated with the β1 integrin

To determine if protein kinase activity was present in the material immunoprecipitated by an mAb against the β1 integrin subunit, P5D2, we assayed immunoprecipitates from unlabeled cells for their ability to incorporate [γ-^{32}P]ATP into precipitated proteins as described in Section 2. Under the conditions tested, no protein kinase activity was detected in material immunoprecipitated by mAb P5D2 when cells were solubilized with NP-40, although the β1 integrin was immunoprecipitated as expected (data not shown). However, when cells were solubilized in a buffer containing Brij 58 as described in Section 2, protein kinase activity was detected in the material immunoprecipitated by mAb P5D2 (Fig. 1). When [γ-^{32}P]ATP was added to the material immunoprecipitated from PA-1 cells by mAb P5D2 (Fig. 1, lane A), ^{32}P was incorporated into a distinct protein of ~50-kDa (arrow). Another mAb to the β1 integrin subunit, P4C10, gave similar results (not shown). The 50-kDa protein was not present when material was immunoprecipitated by normal mouse IgG (lane B). Immunoprecipitates using mAb OC-125 against the ovarian carcinoma antigen CA-125, and an mAb to the c-neu protein did not contain detectable protein kinase activity (not shown). Similar results were obtained with the OVCA 433, SKOV3, CAOV3, and SW626 ovarian carcinoma cell lines in that a ~50-kDa ^{32}P-labeled protein was identified by the protein kinase assay in immunoprecipitates using the anti-β1 mAb (lanes C, E, G, and I) that were not seen in the corresponding immunoprecipitates using normal mouse IgG (lanes D, F, H, and J). In two cell lines, OVCA 433 and CAOV3, a second ^{32}P-labeled protein of ~46 kDa was also detected in the immunoprecipitate using the anti-β1 mAb (lanes C and G). This 46-kDa protein was also observed in the immunoprecipitate using normal mouse IgG, but was of much lower intensity (lanes D and H). With longer exposures, other ^{32}P-labeled proteins were detected in some experiments, but these were not reproducibly observed.

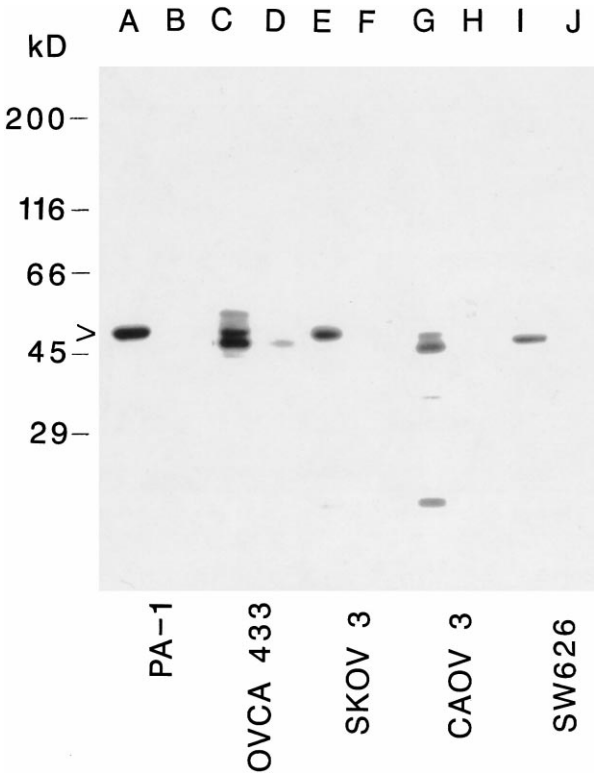

Fig. 1. Co-immunoprecipitation of protein kinases with the β1 integrin. The cell lines PA-1 (lanes A and B), OVCA433 (lanes C and D), SKOV3 (lanes E and F), CAOV3 (lanes G and H), and SW626 (lanes I and J), were solubilized in Brij solubilization buffer and immunoprecipitated with an mAb against the β1 integrin subunit or normal mouse IgG. The immunoprecipitates were incubated with [γ-^{32}P]ATP as described in the text. The resulting phosphoproteins were resolved by SDS-PAGE and visualized by autoradiography. The immunoprecipitating antibodies were either the anti-β1 integrin mAb P5D2 (lanes A, C, E, G, and I) or normal mouse IgG (lanes B, D, F, H, and J). A duplicate experiment gave similar results. Proteins used as molecular weight standards were: myosin heavy chain, 200 000 Da; *Escherichia coli* β-galactosidase, 116 000 Da; bovine serum albumin, 66 000 Da; ovalbumin, 45 000 Da; and carbonic anhydrase, 29 000 Da. The arrow indicates the 50-kDa phosphoprotein that was identified specifically in immunoprecipitates using the anti-β1 integrin mAb.

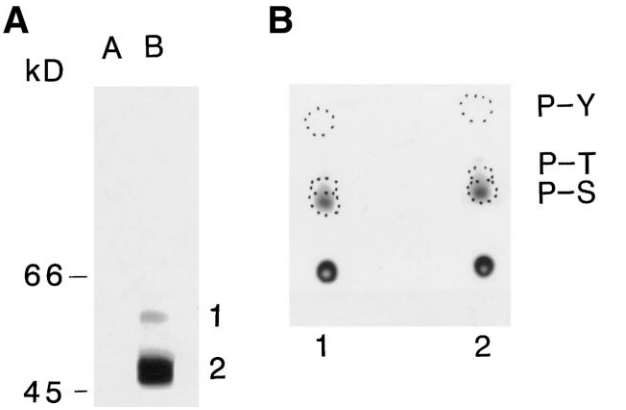

Fig. 2. Phosphoamino acid analysis of proteins co-precipitated with the β1 integrin and labeled in an in vitro kinase assay. PA-1 cells were solubilized in Brij solubilization buffer and immunoprecipitated with 2 μg of purified IgG of the anti-β1 integrin mAb P5D2 (panel A, lane B) or normal mouse IgG (lane A), and the immunoprecipitates were incubated with [γ-^{32}P]ATP as described in Fig. 1. The resulting phosphoproteins were resolved by SDS-PAGE, transferred to Immobilon, visualized by autoradiography (panel A), excised, subjected to acid hydrolysis, and the resultant phosphoamino acids resolved by thin-layer chromatography, and visualized by autoradiography as described in the text (panel B). The lane numbers in panel B correspond to the phosphoproteins numbered in panel A. The positions of migration of authentic phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) are indicated by dotted lines. A duplicate experiment gave similar results.

3.3. Phosphoamino acid analysis

To determine the identity of the ^{32}P-labeled amino acid residues in the proteins phosphorylated by the protein kinase activity associated with the β1 integrin, immunoprecipitates from PA-1 cells were prepared on a larger scale and analyzed by the same protein kinase assay as described in Fig. 1. With the appropriate exposure, two ^{32}P-labeled proteins of ~50 and ~58 kDa were detected in the immunoprecipitate using the anti-β1 integrin antibody (Fig. 2, panel A, lane B) that were not present in the immunoprecipitate using normal mouse IgG (lane A). Each of these individual proteins were excised from the gel and examined for phosphoamino acid content. Phosphoamino acid analyses revealed that the majority of radiolabel in each of the proteins was present on serine residues, demonstrating the presence of serine kinase activity (Fig. 2, panel B).

3.4. Identification of serine protein kinases associated with the β1 integrin

Although the molecular weights of protein kinase C (PKC) isozymes are higher than those of the ^{32}P-labeled substrates seen in Fig. 1, reimmunoprecipitation with antisera to specific PKC isozymes was employed to identify potential PKC that coprecipitated with the β1 integrin. The associated kinase assay was performed as in Fig. 1, and the ^{32}P-labeled proteins were dissociated from the immune complexes and reimmunoprecipitated with antisera to specific PKC isoforms as described in Section 2. Reimmunoprecipitation of ^{32}P-labeled proteins generated from immunoprecipitates with the anti-β1 integrin mAb P5D2 demonstrated no detectable PKC α, β, γ, δ, or ε (not shown). The potential presence of PKC isozymes

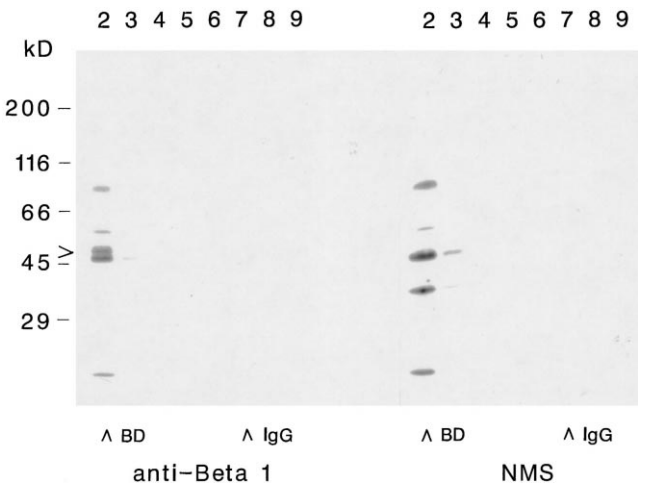

Fig. 3. Determination of the size of the β1 integrin-protein kinase complex. PA-1 extract was fractionated by gel permeation chromatography on Sepharose 4B as described in Section 2. Column fractions were immunoprecipitated with the anti-β1 integrin mAb P5D2 (left panel) or NMS (right panel) and analyzed in the in vitro protein kinase assay as described in Section 2. The arrow indicates the position of the ~50-kDa phosphoprotein detected in the kinase assay shown in Fig. 1. In order to prove that no ~50-kDa protein was visible when immunoprecipitated with NMS, the autoradiograph for NMS (right panel) was exposed for a longer time period than the autoradiograph for the anti-β1 integrin mAb (left panel). With this longer exposure, some ^{32}P-labeled proteins were detected in the NMS panel, but no ~50-kDa protein was detected. The column fractions are indicated at the top. The elution volume of Blue Dextran was fractions 2 and 3, and IgG eluted in fraction 7. Molecular weight standards were the same as in Fig. 1.

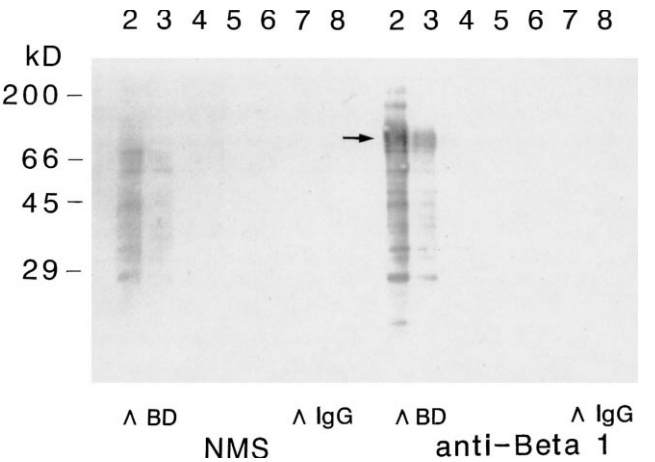

Fig. 4. Distribution of the β1 integrin in gel permeation chromatography fractionated PA-1 cell extract. PA-1 cells were surface labeled with biotin, solubilized in Brij solubilization buffer, and the extract was fractionated by gel permeation chromatography on Sepharose 4B as described in Section 2. Column fractions were immunoprecipitated with the anti-β1 integrin mAb P5D2 (right panel) or NMS (left panel), analyzed by SDS-PAGE under reducing conditions, and the surface labeled proteins detected as described in Section 2. The column fractions are indicated at the top. Blue Dextran eluted in fractions 2 and 3, and IgG in fraction 7. Molecular weight standards were the same as in Fig. 1. The small arrow indicates the position of the β1 integrin subunit.

associated with the β1 integrin was also examined by immunoblotting of immunoprecipitates obtained with the anti-β1 integrin mAb. Again, no PKC α, β, γ, δ, or ε was detected associated with the β1 integrin while PKC α, δ, and ε were readily detected in the cell extract (not shown).

3.5. Gel filtration chromatography

Recent reports suggest that some cell surface proteins may exist in detergent resistant complexes that can be resolved by

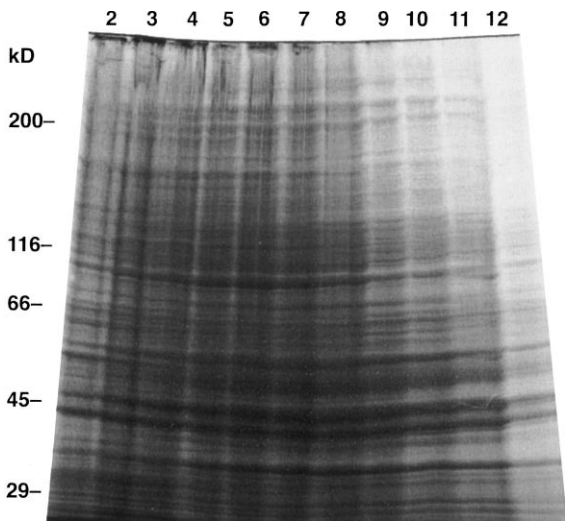

Fig. 5. Distribution of the total cell protein in gel permeation chromatography fractionated PA-1 cell extract. PA-1 cells were solubilized in Brij solubilization buffer, and the extract was fractionated by gel permeation chromatography on Sepharose 4B. Column fractions were analyzed by SDS-PAGE under reducing conditions, and the proteins detected by Coomassie Blue staining. The column fractions are indicated at the top. Molecular weight standards were the same as in Fig. 1.

gel filtration chromatography [15,20,30]. To determine if the β1 integrin-protein kinase complex has similar properties, an extract of PA-1 cells was fractionated on a Sepharose 4B column as described in Section 2. Protein kinase activity in material immunoprecipitated by the anti-β1 integrin mAb P5D2 was detectable only in the void volume fractions 2 and 3 (Fig. 3, left panel). The ~50-kDa ^{32}P-labeled protein observed in Fig. 1 is indicated by the arrow and was not detected in immunoprecipitates using NMS (right panel), even when these autoradiographs were exposed for a long period of time. To determine the distribution of total cell surface β1 integrin in this system, PA-1 cells were surface-labeled with biotin, fractionated by gel filtration chromatography as in Fig. 3, and immunoprecipitated with the anti-β1 integrin mAb P5D2 (Fig. 4, right panel) or NMS (left panel). Biotin-labeled β1 integrin was detected exclusively in the void volume fractions 2 and 3 (small arrow). In contrast, total cell protein as determined by Coomassie Blue staining was detected in fractions 2 through at least fraction 12 (Fig. 5). Since the exclusion limit of Sepharose 4B is more than ~20 million MW, most cell-surface β1 integrin and its associated serine kinase activity appears to be present in complexes of relatively large size.

4. Discussion

This study demonstrates that serine protein kinase activity is associated with the β1 integrin subunit in five ovarian cancer cell lines. This association was specific since control antibodies to other unrelated cell surface molecules did not co-immunoprecipitate the protein kinase activity. This protein kinase activity remained associated with the β1 integrin when cells were solubilized in Brij 58. However, the association of this kinase with the β1 integrin could be disrupted with high concentrations of NP-40, since mAbs to the β1 integrin could not co-immunoprecipitate the protein kinase activity in this detergent. Gel filtration of soluble extract of cells solubilized in Brij 58 demonstrated that both the protein kinase activity associated with the β1 integrin and the majority of the cell surface β1 integrin were found in large detergent-resistant complexes. The majority of ^{32}P incorporated into proteins in the β1 integrin immunoprecipitate using the associated kinase assay was present in a ~50-kDa protein, although longer exposure of the autoradiograph demonstrated an additional ~58-kDa protein. Phosphoamino acid analysis of these phosphoproteins revealed that most of the ^{32}P was incorporated in serine residues.

A novel β_1-integrin linked protein kinase (ILK) was recently identified by using a two-hybrid screen to isolate genes from a human placental cDNA library that interact with the β_1-integrin cytoplasmic domain [32]. This kinase is a ~59-kDa serine/threonine kinase that contains four ankryn-like repeats, and has been reported to phosphorylate a β_1-integrin cytoplasmic domain peptide in vitro on serine and threonine residues to approximately the same degree [32]. Over-expression of this ILK inhibited epithelial cell adhesion to the integrin substrates laminin, fibronectin, and vitronectin, and induced anchorage independent growth in soft agar, suggesting that this ILK is involved in β_1 integrin-mediated signal transduction [32]. Phosphoamino acid analysis of myelin basic protein phosphorylated by ILK and autophosphorylated ILK detected only phosphoserine [32]. These findings support our observation that a serine protein kinase is specifically associated with the β_1 integrin subunit. Whether the 50- or 58-kDa protein detected in the current study has the protein kinase activity, while the other serves as substrate, is not known. However, the higher molecular weight protein detected in the current study is very similar in molecular weight to the ILK (~59 kDa) and may represent the same protein kinase.

Our results also show that, although PKC is present in these cells and PKC isoenzymes have been implicated in signaling pathways for many external signals including extracellular matrix associated proteins, PKC isozymes do not appear to contribute to the β1 integrin associated kinase activity detected here. This was supported by the use of anti-PKC-isozyme specific antibodies on Western blots of proteins immunoprecipitated by antibodies to the β1 integrin. In addition, in re-immunoprecipitation experiments no phosphorylated proteins could be detected using anti-PKC isozyme specific antibodies in material immunoprecipitated by anti-β_1 integrin antibodies and radiolabeled with [γ-^{32}P]ATP in the associated kinase assay.

The finding that both the β_1 integrin associated protein kinase activity and the majority of cell-surface β_1 integrin subunits are present in large detergent-resistant complexes, suggests that they may be present in unique or specialized domains of the plasma membrane. A number of examples of interactions between protein kinases and the cytoplasmic domains of transmembrane proteins have been identified, for example the interaction of CD66a with pp60^{src} via the SH2 domain of pp60^{src} [34]. Protein kinases have also been found to associate with glycosyl-phosphatidylinositol (GPI)-anchored proteins [14,15,20,35–37]. The mechanism whereby protein kinases associate with GPI-anchored proteins is unclear, however, a number of GPI-anchored proteins have been found to be present in large detergent resistant complexes that also contain protein kinases [15–20,22,30,38,39]. For example, the urokinase plasminogen activator receptor (uPA-R) in monocytes has been found to be present in a large receptor complex containing CD18 (β_2 integrin) and the src kinases fyn, lyn, hck, and fgr [30]. Furthermore, the data suggest that more than one type of large complex containing different GPI-anchored proteins exists in monocytes [30]. The concentration and distribution of membrane receptors and signaling molecules in large complexes may be important for signal transmission [30]. The molecular nature of these large detergent resistant complexes is not known, although they may represent membrane microdomains of differing lipid composition [22,38,39]. The present study demonstrates that the protein kinase activity associated with the β_1 integrin in five ovarian cancer cell lines, like the uPA-R in monocytes, is present in large detergent resistant complexes. In addition, the vast majority of cell surface β_1 integrin, as identified by biotin labeling, was also present in large detergent resistant complexes.

Although integrins appear to mediate signals from both the extracellular matrix (ECM) into the cell 'outside-in', as well as from within the cell to the surface 'inside-out' in terms of integrin affinity for ECM substrates, the detailed mechanism of these inside-out and outside-in signaling pathways is not clear. However, protein phosphorylation is an important mechanism of regulation of protein function, and protein phosphorylation has been implicated in integrin mediated signaling. A tyrosine phosphorylation consensus sequence is

present in the cytoplasmic tail of the integrin β subunits [33]. Due to the transmembrane structure of integrins, they are capable of linking the extracellular matrix components with intracellular molecules and thereby mediating the attachment, spreading, and migration of cells. Since integrins also interact with the signal transduction apparatus, the engagement of integrins can result in the release of lipid second messengers, activation of protein kinases, and changes in intracellular calcium and pH. Integrin signaling can also regulate cell proliferation, gene expression, differentiation, and apoptosis. The exact mechanism by which integrins are capable of transmitting signals and how cells integrate these signals with others from their environmental milieu remains to be determined.

In summary, the data suggest that the association of protein serine kinase activity with the β_1 integrin in large detergent resistant complexes may be involved in signal transmission by β_1 integrins. Additional studies addressing the role of this protein kinase in mediating β1 integrin cell signaling, and the nature and identity of the enzyme(s) and of the protein substrates are currently being investigated.

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